THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 17, Issue of April 29, pp. 12456–12461, 1994
Printed in U.S.A.

Heparin Modulates the Interaction of $VEGF_{165}$ with Soluble and Cell Associated *flk-1* Receptors*

(Received for publication, July 8, 1993, and in revised form, February 3, 1994)

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The 165-amino acid form of vascular endothelial growth factor ($VEGF_{165}$) is a mitogen for vascular endothelial cells and a potent angiogenic factor. Expression of a chimeric receptor containing the extracellular domain of the *flk-1* receptor fused to the transmembrane and intracellular domains of the human c-*fms* receptor in NIH-3T3 cells, resulted in the appearance of high affinity binding sites for ^{125}I-$VEGF_{165}$ on transfected cells. The binding of ^{125}I-$VEGF_{165}$ to the *flk-1/fms* chimeric receptor of the transfected cells as well as the $VEGF_{165}$-induced autophosphorylation of the chimeric receptors were inhibited in the presence of low concentrations of heparin (1–10 μg/ml). In contrast, similar concentrations of heparin potentiated the binding of ^{125}I-$VEGF_{165}$ to the endogenous VEGF receptors of the transfected cells, indicating that to some extent, the effect of heparin on ^{125}I-$VEGF_{165}$ binding is receptor type-dependent.

A soluble fusion protein containing the extracellular domain of *flk-1* fused to alkaline phosphatase (*flk-1*/SEAP) was used to study the effects of heparin on the binding of ^{125}I-$VEGF_{165}$ to *flk-1* in a cell-free environment. The fusion protein specifically inhibited $VEGF_{165}$-induced proliferation of vascular endothelial cells, but bound ^{125}I-$VEGF_{165}$ inefficiently in the absence of heparin. Addition of low concentrations of heparin or heparan sulfate (0.1–1 μg/ml) resulted in a strong potentiation of ^{125}I-$VEGF_{165}$ binding, whereas higher heparin or heparan sulfate concentrations inhibited the binding. The effect of heparin on the binding of ^{125}I-$VEGF_{165}$ to *flk-1*/SEAP could not be mimicked by desulfated heparin or by chondroitin sulfate. Both bFGF and aFGF inhibited the binding when low concentrations of heparin were added to the binding reaction. However, higher concentrations of heparin abolished the inhibition, indicating that the inhibition is probably caused by competition for available heparin. Taken as a whole, these results indicate that heparin-like molecules regulate the binding of $VEGF_{165}$ to its receptors in complex ways which depend on the heparin binding properties of $VEGF_{165}$, on the specific VEGF receptor type involved, and on the amount and composition of heparin-like molecules that are present on the cell surface of VEGF receptor containing cells.

Vascular endothelial growth factor (VEGF)[1] is a secreted heparin-binding glycoprotein that displays some structural homology with PDGF. Five forms of human VEGF mRNA encoding VEGF proteins of 121, 145, 165, 189, and 206 amino acids are produced from a single gene as a result of alternative splicing (1, 2). The best characterized VEGF species is the 165-amino acid long form ($VEGF_{165}$). The active form of $VEGF_{165}$ is a homodimer of 47 kDa (1, 3) that induces angiogenesis and blood vessel permeabilization *in vivo* and displays a mitogenic activity that seems to be restricted to vascular endothelial cells (1, 4–8). Several recent reports indicate that VEGF may play an important role in the process of tumor angiogenesis (9–11). $VEGF_{165}$ binds to specific cell surface receptors which are found on vascular endothelial cells, and on several types of non-endothelial cells such as NIH-3T3 cells and melanoma cells which do not seem to respond to $VEGF_{165}$ with a mitogenic response (12–16). Cell surface-associated heparin-like molecules are required for the interaction of $VEGF_{165}$ with the three VEGF receptor types observed on vascular endothelial cells (15) and with the two receptor types present in WW94 melanoma cells (16).

The protein encoded by the *flt* gene was recently reported to be a VEGF receptor (17, 18), belonging to the PDGF receptor subfamily of the tyrosine kinase receptors (19). The *flk-1* gene was isolated from an embryonic liver-derived cell population enriched with primitive hematopoietic stem cells (20, 21) and encodes a tyrosine kinase receptor that is homologous to *flt*. The product of the *flk-1* gene and the product of its human homologue KDR also bind VEGF and undergo autophosphorylation in response to VEGF. However, it is not clear yet whether *flk-1* or *flt* can transduce a VEGF induced mitogenic signal (22, 23). Both *flt* and *flk-1* contain seven immunoglobulin-like loops in their extracellular domains, whereas other members of the PDGF receptor family such as c-*kit* or c-*fms* contain only five immunoglobulin-like loops (20).

We have expressed the *flk-1* cDNA and a chimeric gene containing the extracellular domain of *flk-1* and the tyrosine kinase domain encoded by the c-*fms* gene in NIH-3T3 cells, and we report that both the chimera and the native receptor encoded by the *flk-1* gene bind $VEGF_{165}$ with high affinity. We show that heparin concentrations that inhibit the binding of ^{125}I-$VEGF_{165}$ to the chimeric receptor, and the $VEGF_{165}$-induced autophosphorylation of the chimeric receptor, potentiate the binding of $VEGF_{165}$ to endogenous receptors of NIH-3T3 cells. We also present evidence indicating that the binding of

* This work was supported by Grant 90-00477 from the United States-Israel Binational Foundation, by a grant from the Israel Cancer Research Fund, and by a grant from the Israel Ministry of Science joint program with Germany. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: VEGF, vascular endothelial growth factor; $VEGF_{165}$, 165-amino acid form of vascular endothelial growth factor; aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; CSF-1, colony stimulating factor 1; EGF, epidermal growth factor; PAGE, polyacrylamide gel electrophoresis; SEAP, secreted alkaline-phosphatase.

$VEGF_{165}$ to a soluble fusion protein containing the entire extracellular domain of the *flk-1* receptor is strongly enhanced by heparin, but not by chondroitin sulfate or desulfated heparin.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant $VEGF_{165}$ was purified from the conditioned medium of Sf-9 insect cells infected with a baculovirus-based expression vector for $VEGF_{165}$ as described (24). The factor was highly purified as determined by SDS-PAGE chromatography followed by silver staining, using three purification steps, including hydrophobic chromatography, cation-exchange chromatography, and heparin-Sepharose affinity chromatography. Recombinant human bFGF and aFGF were produced in bacteria as described (15). Recombinant PDGF-BB was kindly given by Dr. I. Vlodavsky (Hadassah-Hebrew University Hospital, Jerusalem). EGF was kindly given by Dr. Gospodarowicz (Chiron Inc.). The pMFG expression vector was kindly given by Dr. Richard Mulligan (Whitehead Institute for Biomedical research, Cambridge, MA). Intestinal mucosa-derived heparin, and chondroitin sulfate A and C, were purchased from Sigma. Oversulfated and desulfated heparin were kindly given by Dr. Svahn (Kabi-Pharmacia Therapeutics, Stockholm, Sweden). Rat liver-derived heparan-sulfate was kindly given by Dr. J. T. Gallagher (Christie Hospital, Manchester, United Kingdom). Suramin was obtained from FBA. Heparin-Sepharose was purchased from Pharmacia. $Na^{125}I$ was purchased from New England Nuclear. Tissue culture plasticware was obtained from Nunc. Tissue culture media, sera, and cell culture supplements were from Beth-Haemek Biological Industries. Prestained high molecular weight size markers were purchased from Bio-Rad. Disuccinimidyl suberate was obtained from Pierce Chemical Co. Anti-alkaline phosphatase antibodies (A-018-01 antibody) were from Medix Biotech. The anti-c-*fms* antibodies were produced against a C-terminal peptide (GDIAQPLLQPNNYQFC). CSF-1 was purchased from Genzyme. All of the other chemicals were purchased from Sigma.

Construction of Expression Plasmids Encoding flk-1/SEAP and flk-1/c-fms Fusion Proteins—An antisense oligonucleotide (5'-GGCAGATCTTTCCAAGTTCGTCTTTTC-3') fusing the last codon (underlined) of the *flk-1* extracellular domain (20) to a *BglI* site (bold) was used in conjunction with a sense *flk-1* oligonucleotide (position 1880 in the *flk-1* sequence) to amplify a small fusion fragment. Digestion of the fragment with *BglI* and *EarI* (position 2452 in the *flk-1* sequence) and gel purification yielded a junction fragment. A 5' *flk-1* fragment was prepared by sequential amplification and joining of the *flk-1* sequences between positions 120 and 1739 from two plasmids followed by the addition of *Hind*III linkers. This fragment was digested with *Hind*III and *SalI* (position 535 after the ATG codon) and gel-purified. A fragment containing the bulk of the extracellular domain was purified following digestion with *SalI* and *EarI*. All of the above fragments were simultaneously ligated with dephosphorylated *Hind*III- and *BglI*-digested Aptag vector (25) and transformed into bacteria.

An oligonucleotide 5'-GGTGCCCAGCATCCCCCGGATGAGTTCCTC-3' joining *flk-1* sequences to c-*fms* at a position six amino acids upstream of the c-*fms* transmembrane domain (26) was synthesized for the construction of the *flk-1/c-fms* (the last *flk-1* amino acid is underlined), and its complement was used to amplify two fragments overlapping at the position of gene fusion. The oligonucleotides used were at positions 1880 of *flk-1* and 2032 of c-*fms*, respectively. Both of these fragments were mixed and re-amplified with the *flk-1* and c-*fms* oligonucleotides (1880 and 2032) to create the fusion fragment. The rest of the cDNA encoding the extracellular domain of *flk-1* was grafted to this fusion fragment as described for the creation of the *flk-1*/SEAP encoding sequence. The sequence encoding the intracellular and transmembrane domains of c-*fms* was grafted to this fusion fragment in a similar manner. The DNA encoding the entire chimera was then subcloned into the pMFG expression vector and expressed in NIH-3T3 cells.

Purification of the flk-1/SEAP Fusion Protein—Ten liters of conditioned medium from *flk-1*/SEAP transfected NIH-3T3 cells were concentrated 10-fold by tangential flow ultra- filtration on a 30-kDa cut-off membrane. Following concentration, the conditioned medium was clarified by filtration through a 0.2-μ filter and then loaded onto a 15-ml monoclonal anti-SEAP-Sepharose column equilibrated previously with 0.1 M Tris-HCl, pH 7.6, 0.5 M NaCl, and 2 mM EDTA. The sample was recirculated over the column five times and then washed with 10 bed volumes of 0.2 M glycine HCl, pH 2.8, 0.5 M NaCl, and fractions were immediately neutralized with 2 M Tris base. Fractions containing purified *flk-1*/SEAP were pooled, dialyzed into 0.01 M phosphate-buffered saline, pH 7.2, and frozen at −20 °C. The purity of the *flk-1*/SEAP preparation was >90% as determined by SDS-PAGE and N terminus sequencing.

Cell Culture—NIH-3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics. Human umbilical vein-derived endothelial cells were maintained, and their proliferation in response to various growth factors monitored, as described previously (3, 27).

Binding and Cross-linking of ^{125}I-$VEGF_{165}$—Iodination of human recombinant $VEGF_{165}$ was performed using the chloramine-T method, as described previously (12). The specific activity of the ^{125}I-$VEGF_{165}$ was about 10^6 cpm/ng. To cross-link ^{125}I-$VEGF_{165}$ to the *flk-1*/SEAP fusion protein, *flk-1*/SEAP (100 ng/ml) and ^{125}I-$VEGF_{165}$ (10 ng/ml) were incubated at room temperature for 1 h in binding buffer containing 10 mM HEPES, 150 mM NaCl, and 20 μg/ml bovine serum albumin (BSA). The cross-linker (disuccinimidyl suberate) was then added to a final concentration of 0.2 mM for 15 min at room temperature, and the reaction was stopped with 20 mM glycine. The binding and cross-linking of ^{125}I-$VEGF_{165}$ to *flk-1* and to *flk-1/c-fms* transfected and nontransfected NIH-3T3 cells were performed, and cross-linked complexes visualized, as described previously (12, 15).

$VEGF_{165}$-induced Autophosphorylation of the flk-1/c-fms Chimeric Receptor in Transfected NIH-3T3 Clone C4 Cells—Confluent cells in a 10-cm dish were transferred to serum-free medium containing 0.05 mM sodium orthovanadate, and the cells were incubated 4 more h in this medium at 37 °C. Subsequently, the medium was changed to serum-free medium containing 0.1% BSA and growth factors were added to the desired concentrations for 1–10 minutes. Following stimulation the medium was aspirated, and the cells were washed quickly with ice-cold Dulbecco's phosphate-buffered saline containing 1 mM sodium orthovanadate. The cells were then lysed with ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 2 mM sodium orthovanadate, 5 mM $ZnCl_2$, 5 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml aprotinin, and 0.1 mg/ml leupeptin). Lysates were transferred to chilled Eppendorf tubes and centrifuged 5 min at 12,000 × *g* at 4 °C. Supernatants from each lysate were incubated with 5 μg of affinity-purified antibody directed against c-*fms* and protein A-Sepharose for 2 h at 4 °C with constant shaking. Beads were subsequently washed once with wash buffer A (10 mM Tris-HCl, pH-8, 0.2% Triton X-100, 150 mM NaCl, 2 mM EDTA, and 1 mM sodium orthovanadate), once more with the same buffer containing 0.5 M NaCl, followed by two washes with 10 mM Tris-HCl pH-8. The beads were boiled in 2 × SDS buffer and the supernatant separated on a 4–12% gradient SDS-PAGE gel. Proteins were transferred to nitrocellulose by electroblotting. Phosphoproteins on the nitrocellulose were detected using an anti-phosphotyrosine antibody (Upstate Biotechnology, Inc.), and bound antibodies were visualized using the ECL system (Amersham).

RESULTS

The Receptor Encoded by the flk-1 Gene Binds ^{125}I-$VEGF_{165}$ with High Affinity—Full-length *flk-1* cDNA was subcloned into the molony murine leukemia virus long terminal repeat driven expression vector pMFG. This expression vector was stably co-transfected into both NIH-3T3 cells and baby hamster kidney-derived fibroblast (BHK-21 cells) (28). The presence of VEGF binding sites was examined in geneticin-resistant clones of cells using ^{125}I-$VEGF_{165}$ binding (12). The transfection resulted in the appearance of clones which expressed low densities (no more than 600 receptors/cell) of high affinity ^{125}I-$VEGF_{165}$ binding sites (not shown). We could not detect a mitogenic response to $VEGF_{165}$ in these cells nor could we detect $VEGF_{165}$-induced autophosphorylation.

To try to overcome these problems we have expressed in NIH-3T3 cells a chimeric cDNA containing the extracellular domain of *flk-1* fused to the transmembrane and intracellular domains of the CSF-1 receptor (c-*fms*). Saturation binding experiments in which increasing concentrations of ^{125}I-$VEGF_{165}$ were bound to NIH-3T3 clone C4 cells expressing the chimeric *flk-1/c-fms* receptors were analyzed by the method of Scatchard using the ligand program (29). These experiments revealed one class of high affinity binding sites for ^{125}I-$VEGF_{165}$ with a dissociation constant of 1.9×10^{-11} M. The density of these *flk-1/c-fms* receptors was 2800 receptors/cell (Fig. 1*B*).

Cross-linking experiments revealed a ^{125}I-$VEGF_{165}$-contain-

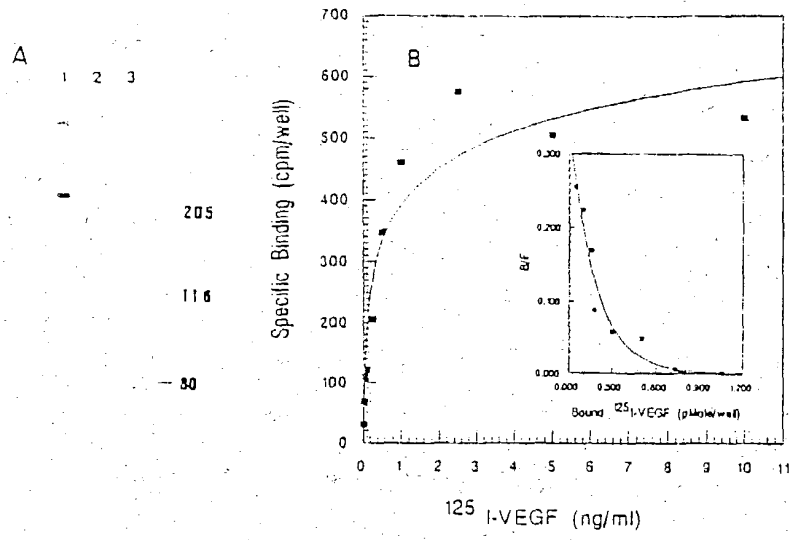

FIG. 1. **Binding and cross-linking of ^{125}I-$VEGF_{165}$ to flk-1/c-*fms*-producing cells.** *A*, expression of the flk-1/c-*fms* chimeric receptor in NIH-3T3 cells. *Flk-1/c-fms*-producing (*lanes 1* and *2*) and nonproducing (*lane 3*) NIH-3T3 cells were grown to subconfluence in 6-cm dishes. The binding of ^{125}I-$VEGF_{165}$ (5 ng/ml) to the cells in the presence (*lane 2*) or absence (*lanes 1* and *3*) of 0.5 μg/ml of unlabeled $VEGF_{165}$ and the subsequent cross-linking of bound ^{125}I-$VEGF_{165}$ to the receptors were conducted as described under "Materials and Methods." *B*, representative saturation binding experiment with flk-1/c-*fms* expressing NIH-3T3 cells. Cells were grown to subconfluence in 24-multiwell dishes (160,000 cells/well). Increasing concentrations of ^{125}I-VEGF, ranging from 10 pg/ml to 10 ng/ml, were bound to the cells for 2 h at 4 °C. Nonspecific binding was measured in the presence of 0.5 μg/ml unlabeled VEGF, and the specific binding was calculated by the subtraction of the nonspecific binding from the total binding. At the end of the binding reaction, the cells were washed three times with 1 ml of ice-cold Dulbecco's phosphate-buffered saline containing 1 mg/ml BSA. The cells were then solubilized with 0.5 ml of 0.2 N NaOH. Aliquots were counted in a γ-counter. Shown is a saturation curve in which the amount of specifically bound ^{125}I-$VEGF_{165}$ is plotted as a function of added ^{125}I-$VEGF_{165}$ concentrations and a Scatchard plot derived from the saturation curve (*inset*).

ing complex of 210 kDa in NIH-3T3 clone C4 cells which is not present in the nontransfected NIH-3T3 cells (Fig. 1*A*, compare *lanes 1* and *3*). The formation of this complex was inhibited by an excess of unlabeled $VEGF_{165}$ (Fig. 1*A*, *lane 2*). The chimeric receptor was autophosphorylated in response to $VEGF_{165}$ in a dose-dependent fashion (Fig. 2). Because of the relatively high expression levels of the *flk-1/c-fms* chimeric receptor in NIH-3T3 clone C4 cells, and because $VEGF_{165}$-induced signal transduction could be observed in these cells, we have chosen to use the NIH-3T3 clone C4 cells for further experiments.

The Effect of Heparin on the Binding of ^{125}I-$VEGF_{165}$ to the Endogenous VEGF Receptors and to flk-1/c-fms Chimeric Receptors Expressed in NIH-3T3 Cells—In the absence of exogenous heparin the endogenous VEGF receptors of the NIH-3T3 cells (15) are barely detectable (Fig. 3, *lane 1*). However, when low concentrations of heparin (1 μg/ml) are included in the binding reaction, two ^{125}I-$VEGF_{165}$-receptor complexes can be detected (Fig. 3, *lane 2*). The binding of ^{125}I-$VEGF_{165}$ to these receptors is inhibited when 0.5 μg/ml of unlabeled $VEGF_{165}$ are added to the binding reaction (not shown). In contrast, the binding of ^{125}I-$VEGF_{165}$ to the *flk-1/c-fms* chimeric receptors expressed in transfected NIH-3T3 clone C4 cells was readily detectable, even in the absence of added heparin (Fig. 1*A* and Fig. 3, *lane 3*). As expected, the endogenous receptors of the transfected cells were not seen when the binding was done in the absence of heparin (Fig. 1*A* and Fig. 3, *lane 3*). No significant effect of heparin on ^{125}I-$VEGF_{165}$ binding to the *flk-1/c-fms* chimeric receptors was observed when heparin concentrations lower than 1 μg/ml were included in the binding reaction (not shown). When 1 μg/ml heparin was added to the binding reaction, the binding of ^{125}I-$VEGF_{165}$ to the *flk-1/c-fms* chimeric receptor was significantly inhibited, whereas the binding of ^{125}I-$VEGF_{165}$ to the endogenous VEGF receptors of the transfected cells was potentiated (Fig. 3, *lane 4*). Similar concentrations of heparin also inhibited partially the $VEGF_{165}$-induced autophosphorylation of the transfected *flk-1/c-fms* chimeric receptors, indicating that the heparin-induced inhibition of $VEGF_{165}$ binding to the transfected receptors is accompanied by reduced receptor function (Fig. 4). A higher concentration of heparin (10 μg/ml) inhibited the binding of ^{125}I-$VEGF_{165}$ to the endogenous and to the transfected VEGF receptors (Fig. 3, *lane 5*). It therefore follows that in the presence of 1 μg/ml heparin the binding of ^{125}I-$VEGF_{165}$ to one type of VEGF receptor is potentiated, whereas the binding of ^{125}I-$VEGF_{165}$ to another class of VEGF receptors is inhibited, although both receptor types reside in the same cell.

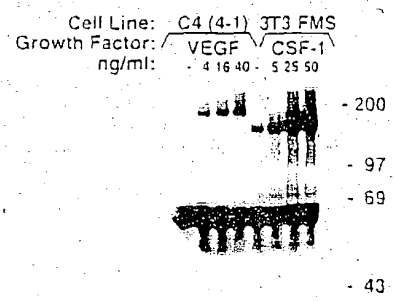

FIG. 2. **$VEGF_{165}$ induces autophosphorylation of flk-1/c-*fms* chimeric receptors.** NIH-3T3 clone C4 and NIH-3T3-*fms* cells were grown in 10-cm dishes and shifted to serum-free medium for 4 h before induction with growth factors as described under "Materials and Methods." The cells were stimulated for 8 min with the indicated concentrations of $VEGF_{165}$ or CSF-1. Following stimulation, the cells were lysed and the receptors immunoprecipitated with anti-c-*fms* antibodies as described under "Materials and Methods." Immunoprecipitated material was chromatographed on a SDS-PAGE gel, transferred to nitrocellulose, and tyrosine-phosphorylated proteins visualized as described under "Materials and Methods."

The Binding of ^{125}I-$VEGF_{165}$ to a Soluble flk-1/SEAP Fusion Protein in a Cell-free Environment Is Potentiated by Heparin—NIH-3T3 cells, as well as most other cell types, contain cell surface-associated heparin-like molecules (30). Therefore the modulatory effects of exogenously added heparin-like mol-

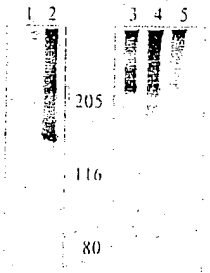

FIG. 3. **The effect of heparin on the binding of ^{125}I-$VEGF_{165}$ to the endogenous VEGF receptors and to the flk-1/c-*fms* chimeric receptors of NIH-3T3 clone C4 cells.** ^{125}I-$VEGF_{165}$ (20 ng/ml) was bound to parental NIH-3T3 cell (*lanes 1* and *2*), and a lower concentration (5 ng/ml) was bound to *flk-1*/c-*fms*-expressing NIH-3T3 clone C4 cells (*lanes 3–5*), in the absence (*lanes 1* and *3*) or in the presence of 1 μg/ml (*lanes 2* and *4*) or 10 μg/ml (*lane 5*) heparin. The subsequent cross-linking of bound ^{125}I-$VEGF_{165}$ to the receptors, and visualization of cross-linked complexes were done as described under "Materials and Methods." *Lanes 1* and *2* were autoradiographed for 9 days, and *lanes 3–5* were autoradiographed for 5 days.

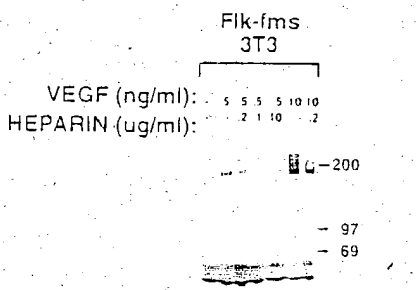

FIG. 4. **Inhibition of $VEGF_{165}$-induced phosphorylation of flk-1/c-*fms* by heparin.** NIH-3T3 clone C4 cells were grown in 10-cm dishes and shifted to serum-free medium for 4 h before the experiment as described under "Materials and Methods." The cells were stimulated with $VEGF_{165}$ at 5 or 10 ng/ml for 8 min in the absence or presence of the indicated concentrations of heparin. Following stimulation, cells were lysed and the receptors immunoprecipitated with anti-c-*fms* antibodies as described under "Materials and Methods." Immunoprecipitated material was chromatographed on a SDS-PAGE gel, transferred to nitrocellulose, and tyrosine-phosphorylated proteins were visualized as described under "Materials and Methods."

ecules on ^{125}I-$VEGF_{165}$ binding are superimposed on the effects of the endogenous cell surface-bound heparin-like molecules. To study the interaction between $VEGF_{165}$ and the *flk-1* receptor in a controlled environment in which the composition of glycosaminoglycans can be controlled precisely, we produced a soluble fusion protein consisting of the extracellular domain of *flk-1* fused to secreted human placental alkaline phosphatase (SEAP). The *flk-1*/SEAP fusion protein was not retained on a heparin-Sepharose column and specifically inhibited the $VEGF_{165}$-induced, but not the basic fibroblast growth factor-induced, proliferation of human umbilical vein-derived endothelial cells by more than 70% (not shown).

To study the binding of ^{125}I-$VEGF_{165}$ to the *flk-1*/SEAP fusion protein, ^{125}I-$VEGF_{165}$ was incubated with the fusion protein and bound ^{125}I-$VEGF_{165}$ was subsequently cross-linked to the soluble receptor. An excess of BSA was added to the binding reaction in order to inhibit nonspecific binding. Labeled ^{125}I-$VEGF_{165}$-*flk-1*/SEAP complexes of 205 kDa could be detected following cross-linking, but the efficiency of complex formation in the absence of heparin was low (Fig. 5*A* and Fig. 5*B*, *lanes 1* and *3*). ^{125}I-$VEGF_{165}$-receptor complexes could be seen under these conditions only after prolonged exposure (not shown). This heparin-independent binding was more prominent when high concentrations of ^{125}I-$VEGF_{165}$ (40 ng/ml) were used (not shown). The addition of 0.1 μg/ml of heparin to the binding

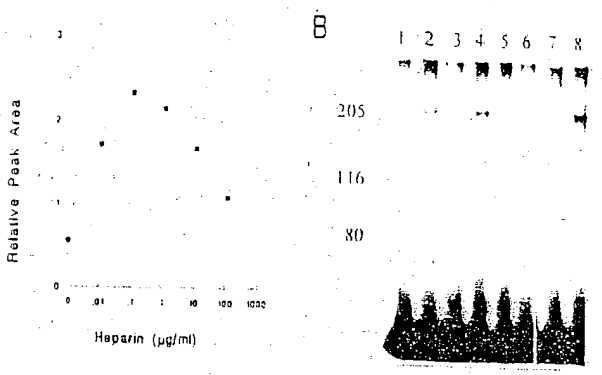

FIG. 5. **The effect of various glycosaminoglycans on the binding of ^{125}I-$VEGF_{165}$ to the flk-1/SEAP fusion protein.** *A*, the effect of increasing concentrations of heparin on the binding of ^{125}I-$VEGF_{165}$ to flk-1/SEAP. ^{125}I-$VEGF_{165}$ (10 ng/ml) was bound to soluble *flk-1*/SEAP (100 ng/ml) in the presence of increasing concentrations of heparin as indicated. Binding was performed for 1 h at room temperature. The subsequent cross-linking of bound ^{125}I-$VEGF_{165}$ to the *flk-1*/SEAP fusion protein and the visualization of cross-linked complexes were done as described under "Materials and Methods." The labeling density of the ^{125}I-$VEGF_{165}$-*flk-1*/SEAP complexes that were formed (see *B*) were determined using a clinicscan-2 densitometer, and plotted as a function of heparin concentration. *B*, the effect of native and modified heparins and of various glycosaminoglycans on the binding of ^{125}I-$VEGF_{165}$ to flk-1/SEAP. ^{125}I-$VEGF_{165}$ (10 ng/ml) was bound to soluble *flk-1*/SEAP (100 ng/ml) in the presence of the following additions: *lanes 1* and *3*, no additions; *lanes 2* and *4*, 0.1 μg/ml heparin; *lane 5*, 0.1 μg/ml chondroitin sulfate A; *lane 6*, 0.1 μg/ml chondroitin sulfate C; *lane 7*, 0.1 μg/ml *N*/*O*-desulfated heparin; *lane 8*, 0.005 μg/ml *O*-oversulfated heparin. Binding was performed for 1 h at room temperature. The subsequent cross-linking of bound ^{125}I-$VEGF_{165}$ to the *flk-1*/SEAP fusion protein and the visualization of cross-linked complexes were done as described under "Materials and Methods."

reaction potentiated the binding of ^{125}I-$VEGF_{165}$ to the soluble receptor (Fig. 5*B*, *lanes 2* and *4*). Densitometric analysis shows that 10 ng/ml of heparin already produce a 3-fold potentiation of ^{125}I-$VEGF_{165}$ binding to the soluble receptor (Fig. 5*A*). A maximal 4-fold potentiation of ^{125}I-$VEGF_{165}$ binding was produced in the presence of 0.1 μg/ml heparin, and heparin concentrations higher than 10 μg/ml progressively inhibited the binding compared with the maximal binding level observed in the presence of 0.1 μg/ml heparin (Fig. 5*A*). Labeled complexes were not formed when ^{125}I-$VEGF_{165}$ monomers were used instead of dimers (12) or when the binding of ^{125}I-$VEGF_{165}$ was performed using SEAP instead of the *flk-1*/SEAP fusion protein (not shown). The heparin concentrations that begin to produce inhibition of ^{125}I-$VEGF_{165}$ binding to the *flk-1*/SEAP fusion protein are higher than the heparin concentrations that are required for partial inhibition of ^{125}I-$VEGF_{165}$ binding to the *flk-1*/c-*fms* chimeric receptors of transfected NIH-3T3 cells (Fig. 3). Thus 1 μg/ml heparin still potentiates strongly ^{125}I-$VEGF_{165}$ binding to *flk-1*/SEAP, but similar concentrations of heparin already inhibit the binding of ^{125}I-$VEGF_{165}$ to the *flk-1*/c-*fms* chimera expressed in transfected NIH-3T3 cells (Fig. 3).

The potentiating effect of heparin appeared to be specific, since identical concentrations of chondroitin sulfate A or chondroitin sulfate C did not produce any enhancement of ^{125}I-$VEGF_{165}$ binding (Fig. 5*B*, *lanes 5* and *6*, respectively). A similar concentration of rat liver-derived heparan sulfate produced a 2-fold potentiation of ^{125}I-$VEGF_{165}$ binding to *flk-1*/SEAP, indicating that the potentiating effects was specific to heparin-like molecules. High concentrations of heparan sulfate (100 μg/ml or more) inhibited even the basal binding of ^{125}I-$VEGF_{165}$ that is observed in the absence of added heparin (not shown). The overall sulfation level of heparin seemed to be critical for the potentiation of ^{125}I-$VEGF_{165}$ binding to *flk-1*/SEAP. When *N*/*O*-desulfated heparin (0.1 μg/ml; total sulfate content, 1%) was

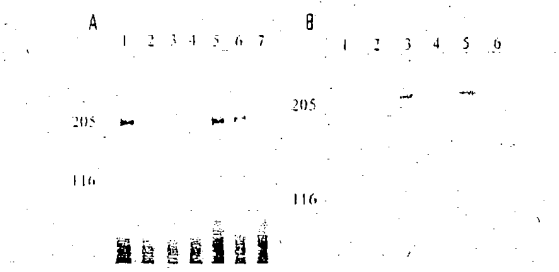

FIG. 6. Specificity of ^{125}I-$VEGF_{165}$ binding to the soluble flk-1/SEAP receptor. *A*, the effect of various growth factors on the binding of ^{125}I-$VEGF_{165}$ to flk-1/SEAP. ^{125}I-$VEGF_{165}$ (10 ng/ml) was bound to the *flk-1*/SEAP protein (100 ng/ml) in the presence of 0.1 μg/ml heparin and in the presence of the following additions: *lane 1*, no additions; *lane 2*, unlabeled $VEGF_{165}$ (0.5 μg/ml); *lane 3*, aFGF (0.5 μg/ml); *lane 4*, bFGF (0.5 μg/ml); *lane 5*, PDGF (0.5 μg/ml); *lane 6*, EGF (0.5 μg/ml); *lane 7*, suramin (1 mM). The binding, the subsequent cross-linking of bound ^{125}I-$VEGF_{165}$ to the *flk-1*/SEAP fusion protein, and the visualization of cross-linked complexes were done as described under "Materials and Methods." *B*, modulation of the inhibitory effect of bFGF by heparin. ^{125}I-$VEGF_{165}$ (10 ng/ml) was bound to the *flk-1*/SEAP protein (100 ng/ml) in the absence of heparin (*lanes 1* and *2*) or in the presence of 0.1 μg/ml heparin (*lanes 3* and *4*) or 1 μg/ml heparin (*lanes 5* and *6*). In addition, bFGF (0.5 μg/ml) was added to some of the binding reactions (*lanes 2*, *4*, and *6*). The binding, the subsequent cross-linking of bound ^{125}I-$VEGF_{165}$ to the *flk-1*/SEAP fusion protein, and the visualization of cross-linked complexes were done as described under "Materials and Methods."

added instead of heparin, no enhancement of ^{125}I-$VEGF_{165}$ binding could be observed (Fig. 5, *lane 7*). When *O*-oversulfated heparin was used (5 ng/ml; total sulfate content, 16%) (31), the enhancement of ^{125}I-$VEGF_{165}$ binding appeared to be more potent than the enhancement achieved with native heparin and was maximal at 5 ng/ml of oversulfated heparin (compare Fig. 5, *lanes 8* and *4*). A higher concentration of *O*-oversulfated heparin (0.1 μg/ml) completely inhibited the binding of ^{125}I-$VEGF_{165}$ to *flk-1*/SEAP, whereas native heparin caused a similar inhibition of binding only when 100 μg/ml were included in the binding reaction (not shown).

The Specificity of the Interaction of the Soluble flk-1/SEAP with $VEGF_{165}$—To examine the specificity of the interaction between ^{125}I-$VEGF_{165}$ and the soluble *flk-1*/SEAP receptor, binding and cross-linking experiments were conducted in the presence of 0.1 μg/ml heparin and various other substances. The binding of ^{125}I-$VEGF_{165}$ to the soluble *flk-1*/SEAP receptor was completely inhibited by 0.5 μg/ml of unlabeled $VEGF_{165}$ (Fig. 6*A*, *lane 2*) and by suramin (Fig. 6*A*, *lane 7*), a known inhibitor of angiogenesis (32) that inhibits the binding of $VEGF_{165}$ to its receptors on vascular endothelial cells (12). The binding of ^{125}I-$VEGF_{165}$ was not inhibited by 0.5 μg/ml of PDGF or by 0.5 μg/ml EGF (Fig. 6*A*, *lanes 5* and *6*). In contrast, aFGF (0.5 μg/ml) (Fig. 6*A*, *lane 3*) and bFGF (0.5 μg/ml) (Fig. 6*A*, *lane 4* and Fig. 6*B*, *lane 4*) inhibited the binding. Because these two factors bind heparin with high affinity, we reasoned that they could perhaps bind free heparin during the binding reaction (33) and therefore make heparin unavailable to ^{125}I-$VEGF_{165}$, resulting in a partial inhibition of ^{125}I-$VEGF_{165}$ binding. This assumption was supported by an experiment in which the binding to the *flk-1*/SEAP fusion protein was done in the presence of increasing concentrations of heparin and 0.5 μg/ml bFGF (Fig. 6*B*). The inhibitory effect of bFGF was abolished when the binding of ^{125}I-$VEGF_{165}$ to *flk-1*/SEAP was done in the presence of 1 μg/ml heparin (Fig. 6*B*, *lane 6*). This experiment indicates that a potent growth factor like bFGF may, under appropriate conditions, serve in the role of a growth inhibitor using a mechanism involving competition for shared cell surface modulators of receptor binding such as heparin-like molecules.

DISCUSSION

The *flk-1* gene encodes a tyrosine kinase receptor and was isolated from a cDNA library prepared from mouse fetal liver-derived cells enriched with primitive hematopoietic stem cells (20, 21, 34). The expression of the full-length *flk-1* cDNA in cells, or the expression of a cDNA encoding a chimeric receptor containing the extracellular domain of *flk-1* fused to the transmembrane and intracellular domains of the CSF-1 receptor (*c-fms*), results in the expression of high affinity binding sites for ^{125}I-$VEGF_{165}$. The affinity of ^{125}I-$VEGF_{165}$ for these receptors was similar to the affinity of $VEGF_{165}$ to VEGF receptors of vascular endothelial cells. Because the chimeric receptor was expressed at higher levels in transfected cells, and because we could show $VEGF_{165}$-induced autophosphorylation of the chimeric receptors, we have used cells expressing the chimeric receptor for further studies.

The binding of ^{125}I-$VEGF_{165}$ to the endogenous VEGF receptors of vascular endothelial cells and to the small number of endogenous VEGF receptors found in NIH-3T3 cells is potentiated by the addition of 1 μg/ml heparin (15). Unexpectedly, both the $VEGF_{165}$-induced autophosphorylation of the chimeric *flk-1*/*c-fms* receptors expressed in the NIH-3T3 clone C4 cells and the binding of ^{125}I-$VEGF_{165}$ to these receptors were inhibited by heparin concentrations equal or larger than 1 μg/ml, whereas lower heparin concentrations had no effect. It follows that heparin can potentiate the binding of ^{125}I-$VEGF_{165}$ to one class of VEGF receptors and inhibit the binding of ^{125}I-$VEGF_{165}$ to another class of VEGF receptors simultaneously. These experiments indicate therefore that the effect that heparin will have on the binding of ^{125}I-$VEGF_{165}$ to specific VEGF receptors depends not only on the heparin binding ability of the growth factor, but to some extent also on specific characteristics associated with specific VEGF receptor types.

It was recently reported that the ligand binding ability of fibroblast growth factor receptor-1 is regulated by the direct binding of heparin to the receptor (35). The effect of heparin on ^{125}I-$VEGF_{165}$ binding could also be in part the result of a direct interaction between heparin and a subset of VEGF receptors. Alternatively, it is possible that heparin modulates the binding of $VEGF_{165}$ to its receptors indirectly through specific cell surface heparin-binding proteins. It was reported that vascular endothelial cells express cell surface heparin receptors (36), and it was shown that cell surface-bound heparin can potentiate the binding of ^{125}I-$VEGF_{165}$ to the VEGF receptors of vascular endothelial cells (15). Taken together, the experiments suggest that the effect of heparin on the interaction of $VEGF_{165}$ with cell surface VEGF receptors is a complex process that needs to be studied using an experimental setup that will allow precise control of the binding environment.

We have taken a step toward the establishment of such an experimental setup by producing a soluble chimeric VEGF receptor containing the entire extracellular domain of *flk-1* fused to soluble alkaline phosphatase (25). This *flk-1*/SEAP fusion protein turned out to be a specific inhibitor of $VEGF_{165}$-induced cell proliferation. This soluble receptor could perhaps be used in the future as an *in vivo* $VEGF_{165}$ antagonist. The availability of the fusion protein allowed us to conduct binding experiments in a precisely regulated cell free environment. The *flk-1*/SEAP-soluble receptor did not bind ^{125}I-$VEGF_{165}$ efficiently in the absence of heparin, but addition of low heparin concentrations (as low as 5 ng/ml) to the binding reaction strongly potentiated the binding of ^{125}I-$VEGF_{165}$ to *flk-1*/SEAP. The effect of heparin on ^{125}I-$VEGF_{165}$ binding could not be mimicked by chondroitin sulfate, but heparan sulfate had a similar effect. The sulfation level of the heparin was important for the potentiating effect, since desulfated heparin had no activity,

whereas oversulfated heparin potentiated the binding of ^{125}I-$VEGF_{165}$ more efficiently than heparin. These experiments, done in a controlled cell free environment, indicate that heparin can directly modulate the binding of ^{125}I-$VEGF_{165}$ to *flk-1*/SEAP and that the modulation in this case is probably mediated exclusively through the interaction of heparin with ^{125}I-$VEGF_{165}$, since *flk-1*/SEAP does not appear to bind to heparin-Sepharose.

In the presence of 1 μg/ml heparin, the binding of ^{125}I-$VEGF_{165}$ to the *flk-1*/c-*fms* receptors on transfected NIH-3T3 cells is inhibited, whereas the same concentration of heparin potentiates the binding of ^{125}I-$VEGF_{165}$ to the soluble *flk-1*/SEAP receptor. Since cell surfaces already contain heparin-like molecules, it follows that the effect of exogenous heparin on the binding of ^{125}I-$VEGF_{165}$ to a cell surface located VEGF receptor will be superimposed upon the effect of pre-existing cell surface associated heparin-like molecules. In addition, putative heparin binding cell surface receptors may also modulate the binding, resulting in complex final effects which are avoided by the usage of the soluble *flk-1*/SEAP receptors.

The binding of ^{125}I-$VEGF_{165}$ to the *flk-1*/SEAP-soluble receptor could not be inhibited by the growth factors PDGF and EGF (6–8). In contrast, high concentrations of bFGF and aFGF inhibited the binding of ^{125}I-$VEGF_{165}$ to the soluble *flk-1*/SEAP fusion protein. We found that these growth factors lost their ability to inhibit the binding of ^{125}I-$VEGF_{165}$ to the soluble *flk-1*/SEAP fusion protein when the binding experiments were conducted in the presence of high concentrations of heparin. It therefore follows that competition for available cell surface heparin-like molecules could perhaps function as an indirect cross-talk mechanism by which a growth factor such as bFGF may modulate the activity of another heparin binding growth factor such as $VEGF_{165}$.

To conclude, our results indicate that heparin affects the interaction of $VEGF_{165}$ with various VEGF receptors by more than one mechanism. We have also shown that heparin can modulate the interaction of $VEGF_{165}$ with the VEGF binding domain of the *flk-1* receptor, even when the binding is done in an environment that contains only *flk-1*/SEAP receptors, VEGF, and heparin. The results of this study indicate that VEGF may play an important role in the maturation process of hematopoietic cells, since the *flk-1* cDNA was originally isolated from a cell population enriched with primitive hematopoietic stem cells (20).

Acknowledgments—We thank Drs. Hela Gitay-Goren and Dina Ron for critically reading this manuscript and for helpful advice and Dr. Carl M. Svahn for the gift of modified heparins.

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AUSTRALIA

Patents Act 1990

IN THE MATTER OF Australian Patent Application Serial No. 696764 by Human Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by Ludwig Institute for Cancer Research

THIS IS Exhibit PAWR-8

referred to in the Statutory Declaration

of Peter Adrian Walton Rogers

made before me

DATED this 12th Day of November, 2001

(Signature of Witness)

Medical Practitioner